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Article

Transportin-SR2 Imports HIV into the Nucleus

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Summary

Background: The human immunodeficiency virus type 1 (HIV-1) and other lentiviruses have the capacity to infect nondividing cells like macrophages. This requires import of the preintegration complex (PIC) through the nuclear pore. Although many cellular and viral determinants have been proposed, the mechanism leading to nuclear import is not yet understood.

Results: Using yeast two-hybrid and pull-down, we identified and validated transportin-SR2 (TRN-SR2) as a bona fide binding partner of HIV-1 integrase. We confirmed the biological relevance of this interaction by RNAi. Depletion of TRN-SR2 interfered with the replication of HIV-1 and HIV-2 but not MoMLV in HeLaP4 cells. Knockdown of TRN-SR2 in primary macrophages likewise interfered with HIV-1 replication. Using Q-PCR, we pinpoint this block in replication to the early steps of the viral lifecycle. A reduction in 2-LTR formation suggests a block in PIC nuclear import upon siRNA-mediated knockdown. Different lines of evidence clearly proved that the late steps of viral replication are not affected. In an in vivo nuclear-import assay using labeled HIV-1 particles, the defect in nuclear import after depletion of TRN-SR2 was directly visualized. In comparison with control cell lines, the great majority of siRNA-treated cells did not contain any PIC in the nucleus.

Conclusion: Our data clearly demonstrate that TRN-SR2 is the nuclear-import factor of HIV.

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Introduction

Lentiviruses such as the human immunodeficiency virus type 1 (HIV-1) have the capacity to infect nondividing cells [1–3]. The viral DNA, packed in the nucleoprotein preintegration complex (PIC), is recognized by the cellular nuclear transport machinery and traverses the nuclear envelope in an active and energy-dependent manner to integrate into human chromatin. Although nuclear import is a critical step in the lentiviral replication cycle and the search for the import factor guiding the PIC into the nucleus has been a long-standing focus of interest, the research field remains highly controversial. In addition to different known cellular import factors, three HIV-1 PIC-associated proteins (matrix [MA], viral protein R [Vpr], and integrase [IN]) have been put forward to play a crucial role in this process (for reviews, see [4, 5]).

HIV-1 integrase (IN) processes and subsequently integrates the viral reverse-transcribed DNA into the host genome. IN was originally claimed to contain a bipartite nuclear localization signal (NLS) in the C-terminal domain [6]. Later studies, however, identified a nonclassical NLS sequence in the catalytic core domain [7]. Classical in vitro import assays with digitonin-permeabilized cells resulted in conflicting hypotheses. Whereas some described an ATP-dependent import mechanism not involving any known karyophylins [8], others accredited the nuclear import of IN to an importin α- or importin α/β-dependent mechanism [6, 7, 9]. However, all different theories have remained ambiguous so far and lack final confirmation in cellular HIV replication experiments.

Instead of studying the nuclear import of isolated viral proteins, Fassati et al. used reverse-transcription complexes (RTCs) in digitonin-permeabilized cells. The inhibition of HIV but not MoMLV replication by siRNA-mediated knockdown of importin 7 (Imp7) suggested a role in mediating the RTC nuclear import [10]. Although these results could not be confirmed in macrophages [11], Imp7 was identified as a cellular binding partner of HIV-1 integrase by coimmunoprecipitation. Even though strong siRNA-mediated knockdown of Imp7 was achieved, only modest effects on HIV-1 replication were observed, leaving space for speculation as to whether Imp7 is indeed a physiological import factor of HIV-1 [12].

Most studies on nuclear import of HIV have been initiated by the question of whether a specific import factor was capable of guiding viral proteins alone or in a complex through the nuclear membrane. In this study, however, we performed a yeast two-hybrid screen (Y2H) in order to identify novel interaction partners of HIV IN. Our special interest was directed toward members of the nuclear-import factor family. We identified transportin-SR2 (TRN-SR2, TNPO3) as a karyophilic interaction partner of integrase.

TRN-SR1 and TRN-SR2 [13, 14] shuttle essential splicing factors, the serine/arginine-rich proteins (SR proteins), between the nucleus and the cytoplasm and therefore are involved in the regulation of mRNA splicing. The recognition of the SR proteins by TRN-SR1 and TRN-SR2 relies on the conserved RS domain and requires phosphorylation [13–15]. Yun et al. [16] showed that TRN-SR1 and TRN-SR2 are encoded by one gene (*tnpo3*) and are expressed via alternative splicing (Figure 1A). In most tissues

and in all cell lines analyzed, only TRN-SR2 was expressed, whereas the TRN-SR1 isoform was undetectable [16]. By yeast two-hybrid screening, multiple non-SR proteins have been identified as binding partners of TRN-SR2. The most prominent binding partner was RNA-binding motif protein 4 (RBM4), a zinc-finger protein involved in splicing regulation [17].

Here, we show the identification and validation of TRN-SR2 as the nuclear-import factor of HIV. Our data indicate that TRN-SR2 mediates the HIV PIC nuclear import and therefore facilitates HIV infection. Because TRN-SR2 is involved in HIV replication in dividing and nondividing cells, we hypothesize that nuclear import of the HIV PIC is required for productive infection.

Results

Yeast Two-Hybrid Screen

In order to identify cellular factors interacting with HIV-1 integrase, we used HIV-YU2 integrase as bait in a yeast two-hybrid screen of a random primed CEMC7 cDNA library [18]. LEDGF/p75, the best characterized cellular cofactor of HIV integration to date [19–24], represented by far the highest number of identified yeast clones and validated the approach. Intriguingly, a nuclear-import factor, transportin-SR2 (Figure 1A), which had not yet been implicated in HIV-1 replication, was identified with four yeast clones. Multiple IN clones were identified in the reverse screen against a library of HIV genome DNA random fragments, demonstrating the exclusivity of the interaction. The selected IN interaction domain (SID) in TRN-SR2 was mapped to the N terminus (aa 62–334). The TRN-SR2 SID in IN was mapped to its catalytic core domain (Figure 1B).

TRN-SR2 Is a Lentiviral-Specific Cofactor of IN

The specificity of the interaction of HIV-1 IN with TRN-SR2 was further analyzed in cellular pull-down assays. Recombinant HIV-1 IN interacted with TRN-SR2 from HeLaP4 cell lysates, whereas MoMLV IN did not interact with TRN-SR2 under these conditions, proving the specificity of the interaction (Figure 1C). This finding was corroborated by in vitro pull-down assays using recombinant TRN-SR2. Even in the absence of any other potential binding partner, a distinct direct interaction of HIV-1 integrase with TRN-SR2 was observed (Figure S1 available online). In contrast to HIV-1, MoMLV is a gammaretrovirus that can only infect dividing cells and is therefore not dependent on passage through the nuclear pore.

TRN-SR2 Knockdown Inhibits HIV Replication

To address the potential role of TRN-SR2 in HIV-1 replication, we analyzed the effect of RNAi-mediated TRN-SR2 knockdown on HIV-1 NL4.3 replication. First, we investigated the effect of transient, siRNA- mediated knockdown of transportin-SR2 in HeLaP4 cells. Therefore, we designed two different siRNAs targeting the 5′ end of the TRN-SR2 transcript (Figure 1A). Efficient knockdown was verified by western blotting of cell extracts obtained at different time points after transfection or by quantitative PCR. Transfection with each siRNA clearly resulted in a pronounced reduction in TRN-SR2 levels, lasting for the duration of the HIV replication experiments (6 days). As expected, transfection with a mismatch siRNA (siTRN-SR_2MM) did not reduce transportin-SR2 levels (Figure 2A). HeLaP4 cells transiently transfected with siRNA targeting TRN-SR2 or the mismatched control were infected with HIV-1 NL4.3 (Figure 2A). In parallel, cells were transfected with an unrelated control siRNA (siGFP) or siCD4, targeting CD4 receptor expression in HeLaP4 cells, as a negative and positive control, respectively. The efficiency of the siCD4-mediated knockdown was confirmed by FACS analysis (data not shown). Twenty-four hours after infection, a pronounced decrease of HIV replication was observed with siTRN-SR_2. After 72 hr of infection, siTRN-SR_2-mediated knockdown inhibited viral replication on average 6-fold when compared to siMOCK (Figure 2A). A similar decrease in replication efficacy was observed after knockdown of the CD4 receptor. The siTRN-SR_2-mediated inhibition was independent of the multiplicity of infection (MOI), precluding an unspecific MOI effect (Figure S2).

In order to rule out virus-strain-specific inhibition, we used different HIV-1 strains (NL4.3 and IIIB) as well as a Raltegravir-resistant NL4.3 strain with two resistance-determining mutations (G140S, Q148H) in the integrase gene [25] (Figure 2B). TRN-SR2 knockdown inhibited all viruses to the same extent. Integrase-inhibitor-resistant viruses are thus still susceptible to TRN-SR2 knockdown. As with HIV-1, TRN-SR2 knockdown inhibited HIV-2 replication 6-fold (ROD, Figure 2B).

Next, the lentiviral specificity of the siTRN-SR_2 effect was investigated. Therefore, HeLaP4 cells transfected with siTRN-SR_2, siCD4 and siTRN-SR_2MM were transduced with VSV-G-pseudotyped HIV-derived lentiviral vectors or MoMLV-derived gammaretroviral vectors. As expected, siCD4 had no effect on the VSV-G-mediated transductions. Whereas lentiviral vector transduction was inhibited 6-fold upon TRN-SR2 knockdown (in comparison with siTRN-SR_2MM), gammaretroviral vector transduction was hardly affected (Figure 2C). In this single-round experiment, CMV-promotor-driven fLuc expression is Tat independent. This points to a block of an early step of HIV replication.

Although siRNA-mediated knockdown provides a powerful tool for target validation, artifacts have to be excluded. Indeed, siRNA may induce unexpected off-target effects in mammalian cells [26]. The use of two distinct siRNAs and the mismatch control has already supported the validity of our data. Still, the transient nature of siRNA-based knockdown in dividing cells might complicate the interpretation of phenotypic effects in infection experiments carried out over a period of several days. Therefore, stable polyclonal and monoclonal knockdown cell lines were generated with lentiviral-vector-encoded small hairpin RNAs (shRNAs) (Figure 3). Knockdown efficiency as determined by qPCR was similar to that obtained after transient knockdown (compare Figures 2A and 3A). TRN-SR2 expression levels may be strictly regulated because this protein is involved in the nuclear import of essential mRNA splicing factors [13–15]. This hypothesis is supported by the finding that overexpression of TRN-SR2 in HeLaP4 cells for a prolonged period of time was not possible. Even under selection, overexpression was lost during passaging of HeLaP4 cells. Moreover we were not able to overexpress TRN-SR2 deletion mutants fused to eGFP to sufficiently high levels to induce transdominant inhibition of HIV replication (data not shown). In cell lines stably expressing short hairpins targeting TRN-SR2, HIV replication was reduced 20-fold (Figure 3A). In addition, viral breakthrough experiments in shTRN-SR_2 polyclonal knockdown cell lines did not yield virus up to 9 days after infection (Figure 3B).

HeLaP4 is an established laboratory cell line for the study of HIV-1 replication, but not a physiological host cell line for HIV. Moreover, nuclear import seems absolutely required for lentiviral infection of nondividing cells. In order to ultimately prove

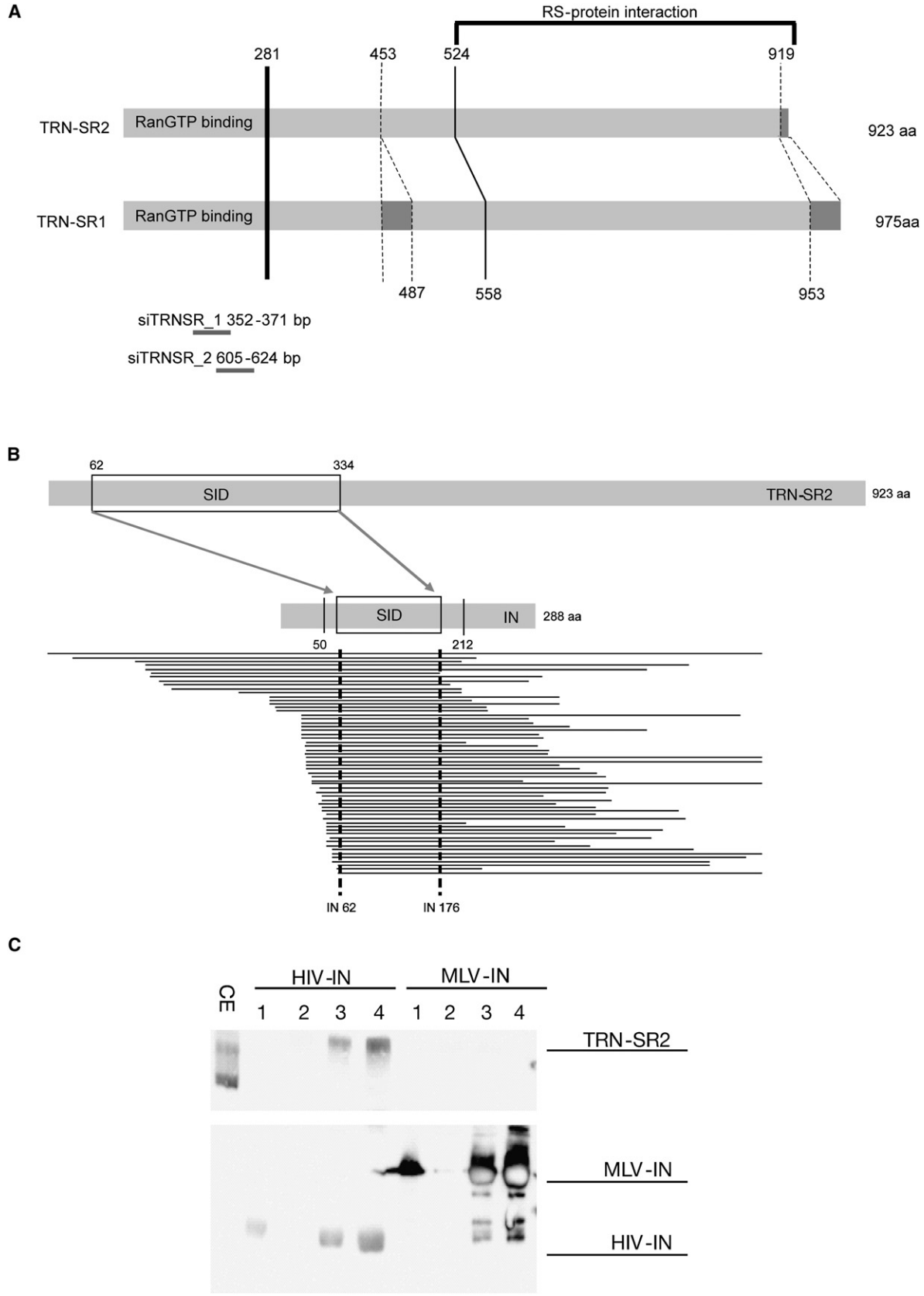

Figure 1. Schematic Representation of TRN-SR and Analysis of Its Interaction with IN

(A) Schematic representation of the TRN-SR1 and TRN-SR2 splice variants of the *tnpo3* gene. Both variants are involved in the nuclear import of serine/arginine-rich proteins (SR proteins). TRN-SR1 is a 975 aa protein consisting predominantly of the RanGTP-binding (aa 1–281) and the RS-protein-binding

the participation of TRN-SR2 in HIV replication, we studied the effect of RNAi-mediated knockdown of TRN-SR2 in primary human macrophages (Figure 4). Macrophages were transfected with siTRN-SR_2 and siTRN-SR_2MM. Comparison of TRN-SR2 expression levels in knockdown macrophages with cells transfected with the mismatched control by qPCR demonstrated a 2-fold reduction. In these conditions and over a period of 8 days, HIV-1 replication was persistently impaired in macrophages transfected with siTRN-SR_2.

TRN-SR2 Knockdown Exclusively Inhibits Early Steps of HIV Replication

Different lines of evidence (transient and stable TRN-SR2 knockdown in laboratory cell lines as well as transient knockdown in macrophages) support the hypothesis that the karyopherin TRN-SR2 is involved in HIV replication. Furthermore, interaction assays demonstrated the direct interaction of HIV IN with TRN-SR2. On the other hand, transportin-SR2 has been implicated in the nuclear import of the essential splicing factor ASF/SF2 [14, 15]. Earlier studies linked this splicing factor to the splicing of the viral pre-mRNA into the multiple-spliced viral mRNAs regulating the Rev, Env, Vpu, and Nef expression [27]. Overexpression of ASF/SF2 in 293T cells altered the splicing pattern of HIV-1 mRNA during infection, resulting in oversplicing of mRNA and therefore inhibiting virus production [28]. Moreover, in the present study, even moderate knockdown of TRN-SR2 had a severe impact on HIV-1 replication (Figures 2A, 2B, 3A, and 4). Accordingly, we wondered whether knockdown of TRN-SR2 exclusively affected early steps of HIV replication or also indirectly affected later steps of the HIV-1 life cycle, augmenting the inhibition of HIV replication.

For evaluation of the impact of siTRN-SR_2-mediated knockdown on early steps of HIV-1 replication, HeLaP4 cells were harvested at different time points after HIV-1 NL4.3 infection. DNA was extracted to quantify late reverse transcripts, 2-LTR circles, and integrated proviruses via qPCR. siMOCK- and siTNR-SR_2MM-transfected cells were used as controls (Figure 5A). Of interest, a 3- to 4-fold reduction in 2-LTR circles was evidenced in TRN-SR2 knockdown cells but not in the mismatch control. This finding correlated with the strength of knockdown as determined by qPCR and the 3- to 5-fold reduction of HIV replication (see Figure 2A). Likewise, proviral DNA was hardly detectable after TRN-SR2 depletion. 1-LTR and 2-LTR circles are formed as dead-end by-products of viral replication [29]. Therefore, the number of 2-LTR circles is an indirect measure for the nuclear import of the PIC. The clear reduction in 2-LTR formation and subsequent block of integration pinpoint the block of HIV-1 replication between late reverse transcription and the nuclear entry step.

Depletion of TRN-SR2 by siRNA did not affect p24 production in HeLaP4 cells transfected with a molecular clone of HIV-1 NL4.3 in comparison with the control cells (siMOCK or siTRN-SR_2MM, Figure 5B). As expected, addition of the protease inhibitor Saquinavir (Roche) completely abolished p24 production. Moreover, the cellular distribution of the physiological TRN-SR2 cargo, the RS-splicing factor ASF/SF2, was not altered upon TRN-SR2 knockdown (Figure 5C). This can be explained by the fact that ASF/SF2 can enter the nucleus during cell division of HeLaP4. Therefore, the inhibition of HIV replication seen by TRN-SR2 knockdown is probably not due to an altered cellular distribution of ASF/SF2. Ultimately, we evaluated the impact of TRN-SR2 knockdown on the expression of multiple-spliced viral mRNA by quantitative RT-PCR. Cell lines depleted of TRN-SR2 were transfected with a molecular clone of HIV-1, NL4.3. No difference in multiple-spliced 1.8 kb viral mRNA was detected between knockdown and control cell lines (Figure 5D). Taken together, all four lines of evidence support the hypothesis that TRN-SR2 is responsible for the nuclear import of the HIV PIC and does not affect late steps in the viral life cycle.

TRN-SR2 Imports the PIC into the Nucleus

Nuclear import of cellular proteins is typically studied with recombinant import factors in digitonin-permeabilized cells. Although such assays exponentially increased our knowledge of nuclear import, they may lack physiological relevance for studying viral import. Through the study of isolated viral proteins instead of the viral PIC, NLS signals of viral proteins may be exposed that are masked within the multiprotein complex and therefore are not accessible for the cellular import machinery. In agreement with earlier reports [7, 8, 10, 12], we found that different import factors stimulated to some extent the import of HIV IN to the nucleus (data not shown). However, to solve the conflicting observations surrounding the nuclear import of HIV [5], we have now applied a cellular nuclear-import assay [30] to corroborate the role of TRN-SR2 in HIV infection. The efficient nuclear translocation of the PIC in HeLaP4 cells was evaluated with fluorescently labeled viral particles containing IN fused to eGFP (HIV-IN-eGFP). IN-eGFP was incorporated in HIV particles by Vpr mediated transincorporation [31]. IN-eGFP was able to functionally complement IN D64E encoded by a proviral construct, resulting in the ability of HIV particles to integrate [30]. Immunostaining of the matrix and capsid proved the identity of fluorescent viral particles (data not shown). For verification of the functionality of the virions, the natural endogenous reverse transcription (NERT) activity was assessed. De novo synthesis of cDNA was demonstrated by incorporation of fluorescent deoxynucleotides in extracellular virions. After infection, eGFP-labeled PICs were associated with fluorescent-labeled cDNA (data not shown). HeLaP4 cells treated with Alexa-568-labeled siRNA directed against TRN-SR2 were infected for 6 hr with HIV-IN-eGFP. In cells positive for Alexa-568, the number of intranuclear and cytoplasmic PICs was determined (Figure 6A). In two independent experiments, the cellular distribution of the PICs was analyzed for siTRN-SR_2- and siTRN-SR_2MM-transfected cells. For each siRNA, 55 or 100 cells were analyzed by confocal microscopy. The average population of eGFP-labeled PICs in the nucleus equaled 2.2% ± 0.1% of all PICs detected in mismatch control cells. This number was similar to the number of PICs observed in the nucleus of nontransfected HeLaP4

domain (aa 558–953). TRN-SR2 (923 aa) is the predominant isoform and is transcribed via alternative splicing. The siRNAs used (siTRN-SR_1 and siTRN-SR_2) are indicated below.

(B) Representation of the selected interaction domains (SID) of TRN-SR2 and IN. Whereas four cDNA clones of TRN-SR2 were selected by yeast two-hybrid screening, multiple clones of HIV IN were identified in the reverse screen.

(C) TRN-SR2 interacts with HIV-1 but not with MoMLV IN (lanes 3 and 4). Pull-down assays were analyzed by western blotting. CE denotes cell extract; lanes 1, loading controls for HIV-IN and MoMLV-IN; lanes 2, pull-downs of CE without adding IN.

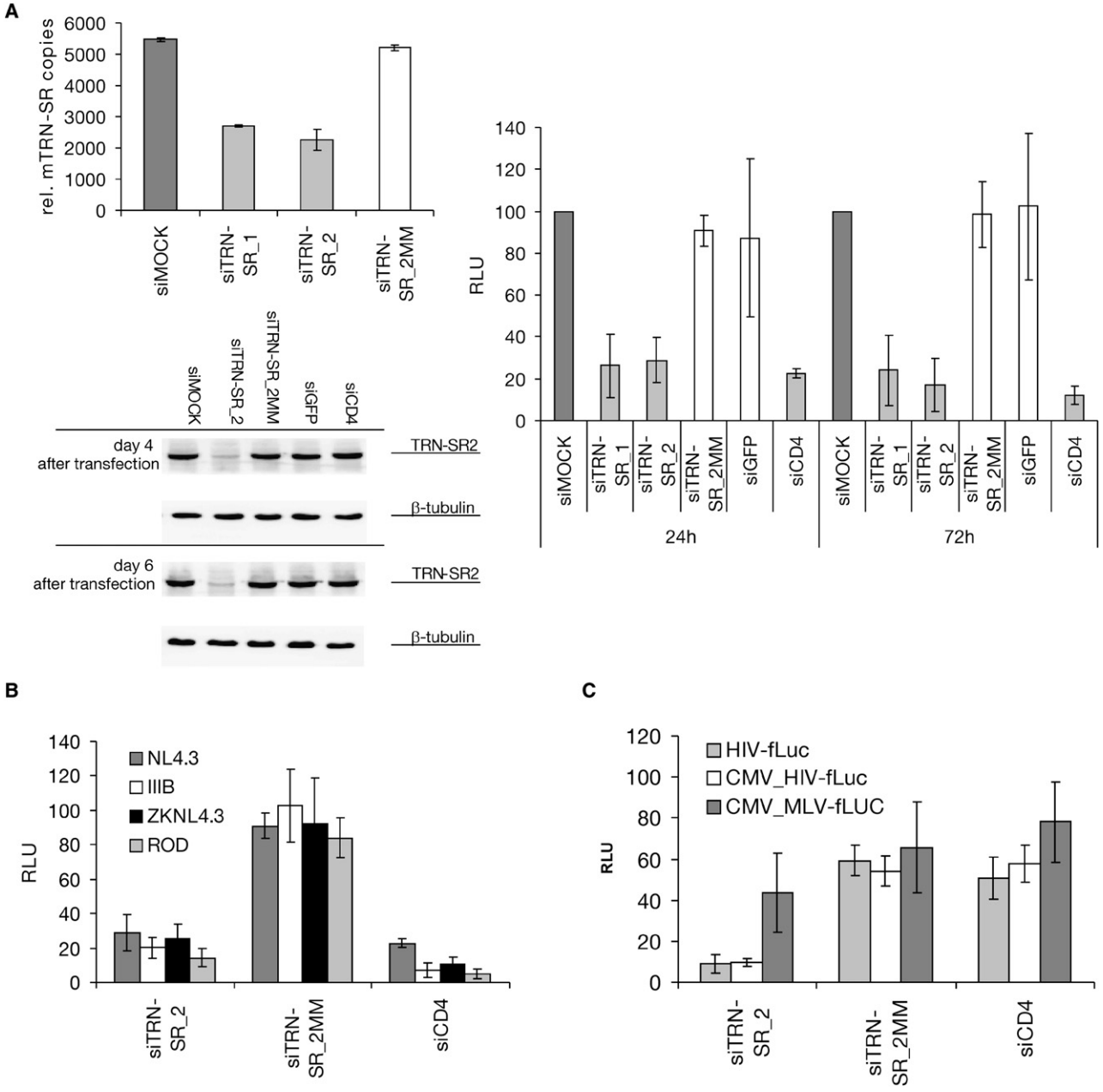

Figure 2. Transient, siRNA-Mediated Knockdown of TRN-SR2 in HeLaP4 Cells Inhibits HIV Replication and Vector Transduction

(A) HIV replication in transient TRN-SR2 knockdown cells. Transfection of each of two different siRNAs targeting the TRN-SR2 mRNA resulted in moderate reduction of the mRNA as determined by qPCR at the day of HIV-1 infection (left panel) (data are represented as mean ± standard deviation [SD]). Western-blot analysis of the knockdown with siTRN-SR_2 showed strong reduction in protein levels lasting at least 6 days (lower left panel). HIV replication was measured by β-galactosidase reporter gene expression (right panel). Transient knockdown of TRN-SR2 resulted in a 6-fold inhibition of HIV-replication in HeLaP4 cells (n = 10, data are represented as mean ± SD). In parallel experiments, neither an unrelated siRNA (siGFP) nor the specific control (siTRN-SR_2MM) suppressed HIV replication upon transfection. Transient knockdown of the CD4 receptor in these cell lines reduced the infectivity of HIV-1 to levels similar to those observed with siTRN-SR_2.

(B) Transient knockdown of TRN-SR2 in HeLaP4 equally inhibited HIV-1 (NL4.3, IIIB, ZKNL4.3) and HIV-2 (ROD) replication as determined by β-galactosidase activity after 24 hr (n = 6, data are represented as mean ± SD).

(C) Inhibition of virus replication after transient knockdown of TRN-SR2 is specific for lentiviruses. HIV-1 expressing the luciferase reporter (HIV-fLuc) and an HIV-1 based vector (CMV_HIV-fLuc) were inhibited 10-fold upon knockdown. In contrast, CMV_MLV-fLuc was not significantly inhibited. As expected, no significant inhibition of replication was seen with the mismatched control and siCD4.

cells [30] and reflects an apparent bottleneck during infection with these eGFP-IN-labeled particles. Strikingly, the nuclear/cytoplasmic ratio dropped 5-fold to 0.4% ± 0.05% in the TRN-SR2 knock down cells. The non-parametric two-tailed Kolmogorov-Smirnov test revealed a statistically highly significant difference ($p < 0.001$) between the two cell populations (Figure 6B). This analysis clearly demonstrates a decrease in the number of viral PICs in the nucleus upon TRN-SR2

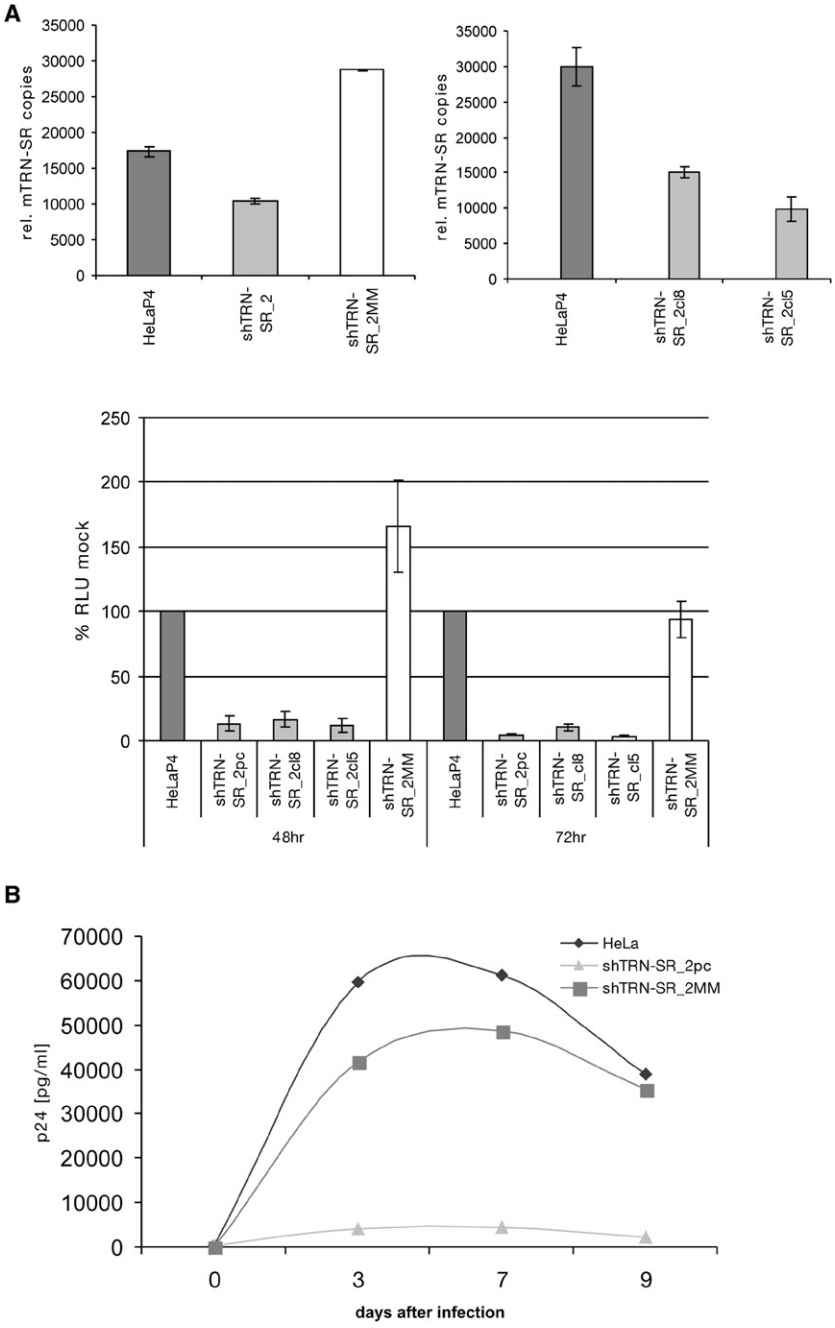

Figure 3. shRNA-Mediated Knockdown of TRN-SR2 Blocks HIV Replication

(A) Polyclonal and monoclonal cell lines encoding shTRN-SR_2 were obtained with moderate reductions of TRN-SR2 expression as determined by qPCR (upper panel). Stable knockdown of TRN-SR2 resulted in a 20-fold reduction of HIV-1 replication as determined by p24 measurement after 24 hr and 72 hr (n = 6, data are represented as relative light units [RLUs] and normalized on the infection of wild-type HeLaP4 cells, mean ± SD is shown). Maximal inhibition was already achieved with the polyclonal cell line; in selected monoclonal cell lines, inhibition of HIV-1 replication was not significantly enhanced (lower panel).

(B) Inhibition of HIV replication in shTRN-SR_2-expressing cell lines was observed over a prolonged time course. Viral breakthrough experiments demonstrated long-term inhibition (9 days) of replication (one representative experiment shown).

depletion and demonstrates a primary role of this import factor in PIC nuclear translocation.

Discussion

Various import factors and viral proteins have been implicated in HIV nuclear import [4, 5]. Here we present the identification and validation of TRN-SR2 as the nuclear-import factor of the HIV PIC. Whereas identification of cellular partners of viral proteins is based on a variety of screening technologies that all have inherent rates of false positives and negatives (e.g., yeast two-hybrid, coimmunopreciptation, and RNAi), validation of biologically relevant interactions requires various lines of additional experimentation. For instance, after the first identification of LEDGF/p75 as a binding partner of HIV-1 integrase in 2003 [19], it took several years and the efforts of various research groups to validate the role of LEDGF/p75 in HIV integration [20–23, 32–38]. After identification of TRN-SR2 as an interaction partner of HIV-IN in yeast two-hybrid, we corroborated the relevance of the interaction by pulling down TRN-SR2 from cell lysates with HIV-1 IN but not with MoMLV IN. Analysis of the clones identified in yeast two-hybrid indicates that TRN-SR2 interacts with the catalytic core domain of IN close to its N terminus. Currently, work is in progress to design and express a set of deletion mutants of both proteins for analysis by confocal microscopy and recombinant protein expression. These mutants will allow us to define the interaction domain in detail. Efforts are also ongoing to identify single-point mutations in IN that inhibit the interaction with TRN-SR2. Given that mutations in IN tend to have pleiotropic effects on HIV replication, conclusions will have to be drawn with care [35, 39].

Here we demonstrated that two short interfering RNAs as well as shRNAs inhibited HIV replication drastically. We proved that TRN-SR2 is a cofactor for various HIV-1 strains and an HIV-2 strain but not for MoMLV, suggesting lentiviral specificity and excluding unspecific off-target effects (Figure 2). Several lines of evidence (qPCR and analysis of the multiple-spliced HIV-mRNA, cellular localization of ASF/SF2, and virus production) indicate that TRN-SR2 knockdown does not affect late steps in the viral replication cycle. Moreover, quantitative analysis of viral DNA species directly pinpointed the block of HIV replication to the nuclear-import step of the PIC (Figure 5). siRNA-mediated knockdown of TRN-SR2 in dividing (HeLaP4) and nondividing (macrophages) cells strongly affected the infectivity of HIV, indicating that the transport through the nuclear envelop might be necessary for productive infection of the host cell.

To determine the molecular mechanism of TRN-SR2 in detail, we studied the nuclear import of the PIC in cells. The

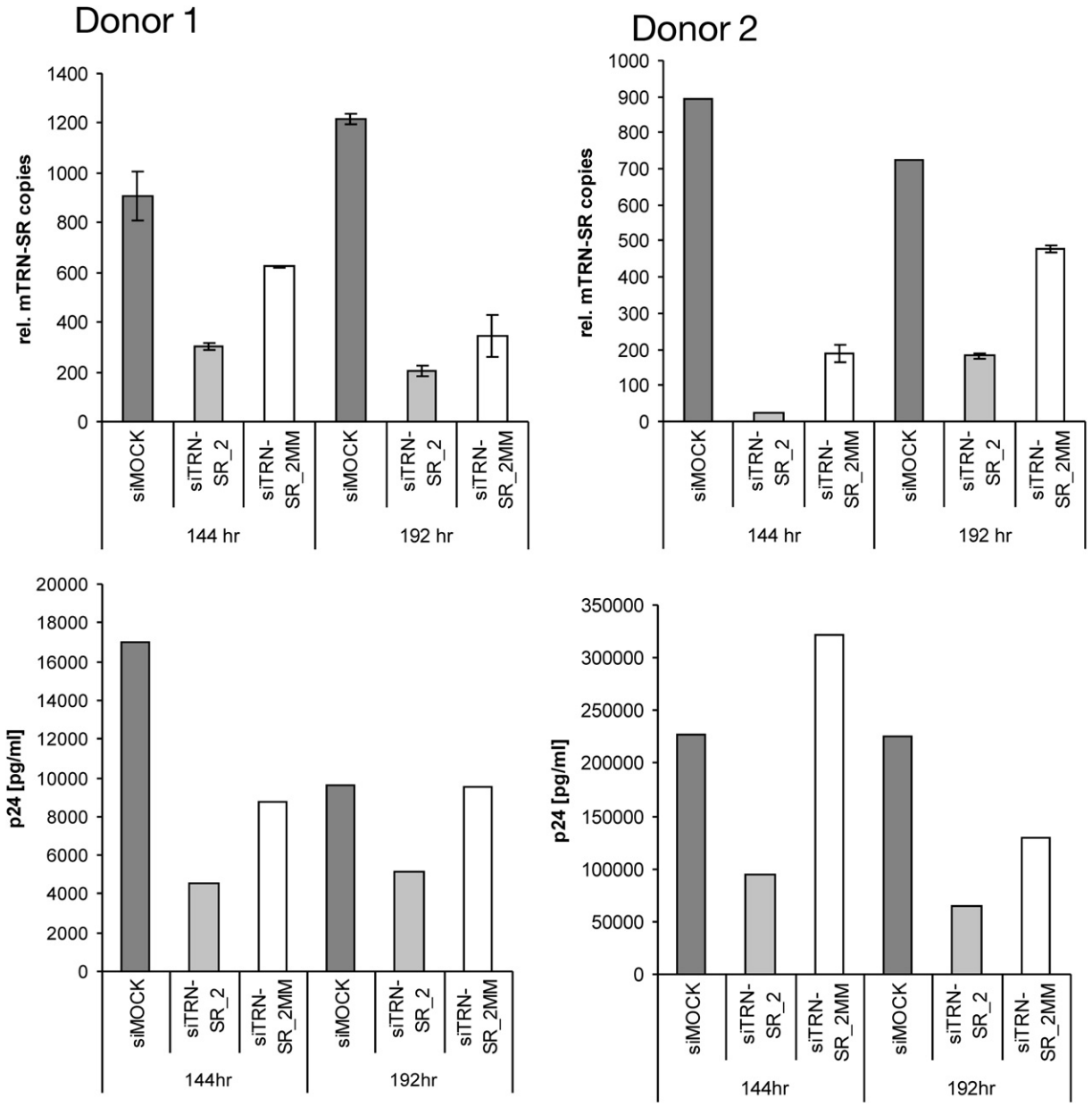

Figure 4. Effect of TRN-SR2 Knockdown in Primary Macrophages

Two independent experiments with cells from different donors are shown. Knockdown of TRN-SR2 by siTRN-SR_2 on the day of infection was analyzed by qPCR. After infection of transiently transfected macrophages with HIV-1 YU2, viral replication was analyzed by p24 measurements in the supernatant. Compared to mock or siTRN-SR_2MM-transfected macrophages, a 2-fold reduction in expression levels resulted in a 2- to 3-fold decrease in HIV replication.

state-of-the-art technique is an in vitro transport system in which recombinant transport factors and their potential cargoes are added to digitonin-permeabilized cells (for a review, see [40]). For HIV, these studies have only provided limited information to date. Because these import assays use recombinant integrase, NLS sequences that are normally hidden in the PIC might get inadvertently exposed. Therefore, it is not surprising that several karyopherins (importin α, importin β, and importin 7) have been put forward as putative import factors of HIV-1 integrase [6, 9, 12]. So far, inhibition of HIV-1 replication after RNAi-mediated knockdown has only been achieved for Imp7 [12]. Notwithstanding potent knockdown of Imp7, only moderate inhibition of HIV replication was achieved, and Zielske et al. could not detect a phenotype in macrophages [11]. Here, we used a novel approach to study HIV nuclear import with fluorescently labeled HIV in living cells [30]. We obtained unambiguous results: Specific knockdown of TRN-SR2 decreased PIC import by more than 5-fold, corroborating TRN-SR2 as the cellular import factor of HIV-1. Although we cannot exclude with absolute certainty the possibility that a subpopulation of the eGFP-labeled particles might not be functional, our data clearly demonstrate that in the control cells a fraction of the particles gets imported into the nucleus. In contrast, siTRN-SR_2-mediated knockdown excludes the labeled PICs from the nucleus. Quantification of the populations of PICs in the cytoplasm and the nucleus

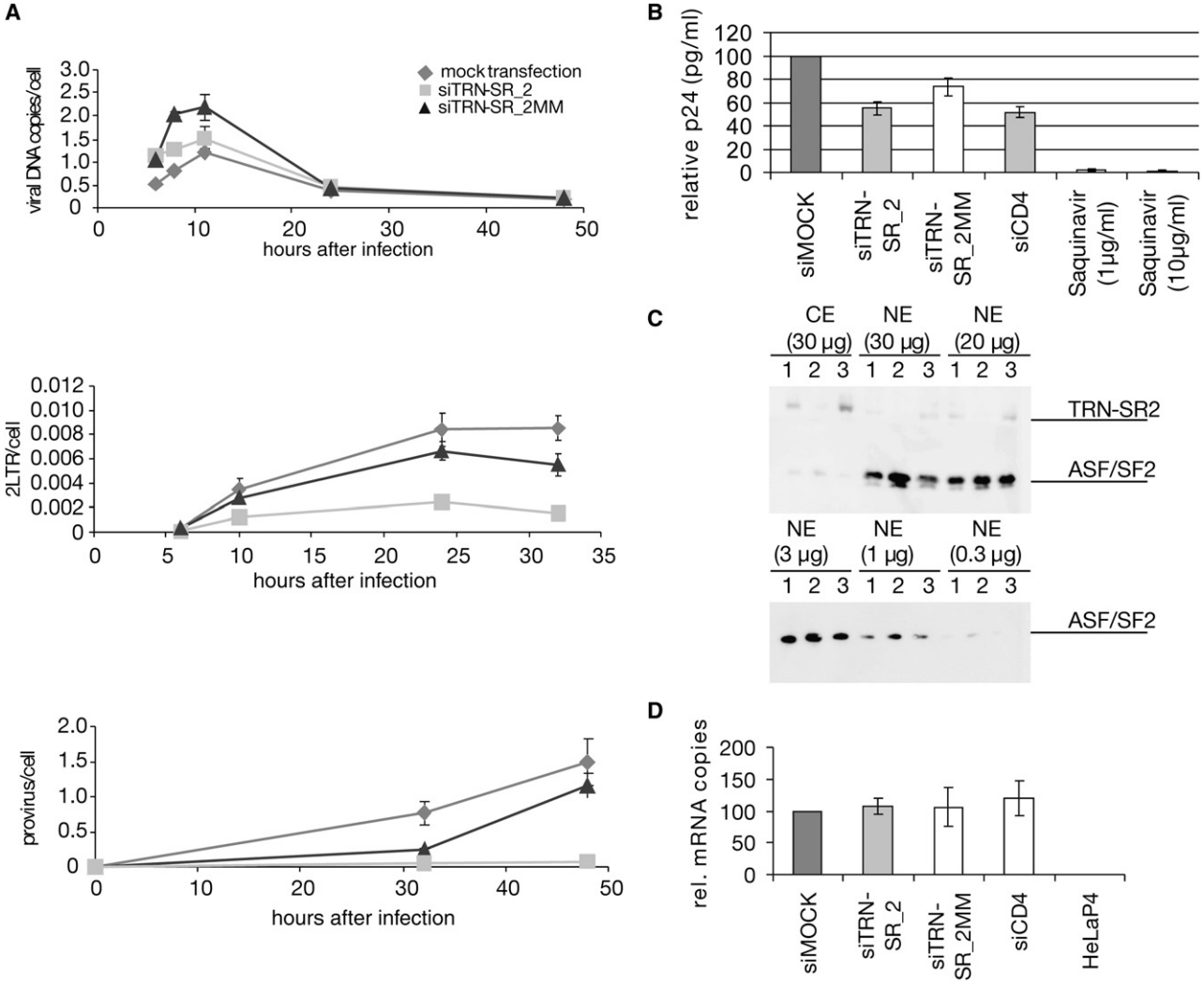

Figure 5. siRNA-Mediated Knockdown of TRN-SR2 Exclusively Inhibits Early Steps of HIV Replication

(A) The block of HIV-1 replication upon knockdown of TRN-SR2 can be pinpointed to nuclear import. After transient transfection of siTRN-SR_2 and the mismatched control, HeLaP4 cells were infected with HIV-1 NL4.3. At distinct time points after infection, DNA was extracted from cells and viral DNA species were identified by qPCR (viral DNA copies, 2-LTR, provirus). Whereas all cells permit reverse transcription of viral RNA, siTRN-SR_2 transfection reduced 2-LTR circles 3-fold, indicating a block in nuclear import of the PIC. Only trace amounts of integrated virus were detectable in those cells.

(B) Cells were transfected with a molecular clone of HIV-1 NL4.3. Production of HIV-1 NL4.3 in siTRN-SR_2 knockdown cells was not hampered, demonstrating that the siRNA does not cause late effects on viral replication. NL4.3 production in cells treated with 1 or 10 μg/ml of the protease inhibitor Saquinavir was severely affected (n = 3, data are represented as mean ± SD).

(C) siTRN-SR_2 does not alter the cellular distribution of ASF/SF2 as determined by western blotting. CE denotes cytoplasmic extract; NE denotes nuclear extract; lanes 1, siMOCK; lanes 2, siTRN-SR_2; lanes 3, siTRN-SR_2MM.

(D) Analysis of multiple-spliced HIV mRNA after transient knockdown of TRN-SR2 and subsequent transfection with a molecular clone of HIV-1 NL4.3 (n = 3, data are represented as mean ± SD). Knockdown of neither TRN-SR2 nor CD4 changed the splicing pattern of the HIV mRNA. The multiple-spliced transcript was exclusively detected in pNL4.3-transfected cells but not in the nontransfected control (HeLaP4).

revealed that nuclear import is a critical rate-limiting step during HIV-1 replication. This may help explain the strong phenotype seen upon knockdown of TRN-SR2 (Figures 2, 3, and 4).

It is well known that gammaretroviruses such as MoMLV cannot productively infect nondividing cells. However, to date it is not clear whether this is due to a defective nuclear import and a concomitant requirement for disassembly of the nuclear membrane during mitosis or whether MoMLV integration requires a cellular cofactor expressed during mitosis. The Emerman group put forward the hypothesis that the amount of capsid (CA) protein present in the viral PIC is the major determinant for the lack of infection of nondividing cells [41, 42]. In contrast to HIV, the MoMLV PIC contains high amounts of CA. Our data indicate that TRN-SR2 acts as a cofactor of HIV nuclear import in both dividing and nondividing cells and suggest a lack of interaction between TRN-SR2 and MoMLV IN. Whether p24 plays a role in TRN-SR2-mediated HIV nuclear import awaits further experimentation.

While this manuscript was in preparation, Brass et al. published an extensive siRNA screen to identify host factors of HIV-1 IIIb replication [43]. Two hundred and seventy-three siRNAs that decreased HIV replication at least 2-fold were identified. Interestingly, the *tnpo3* gene encoding for TRN-SR2 was identified as a hit. Although this report did not pinpoint

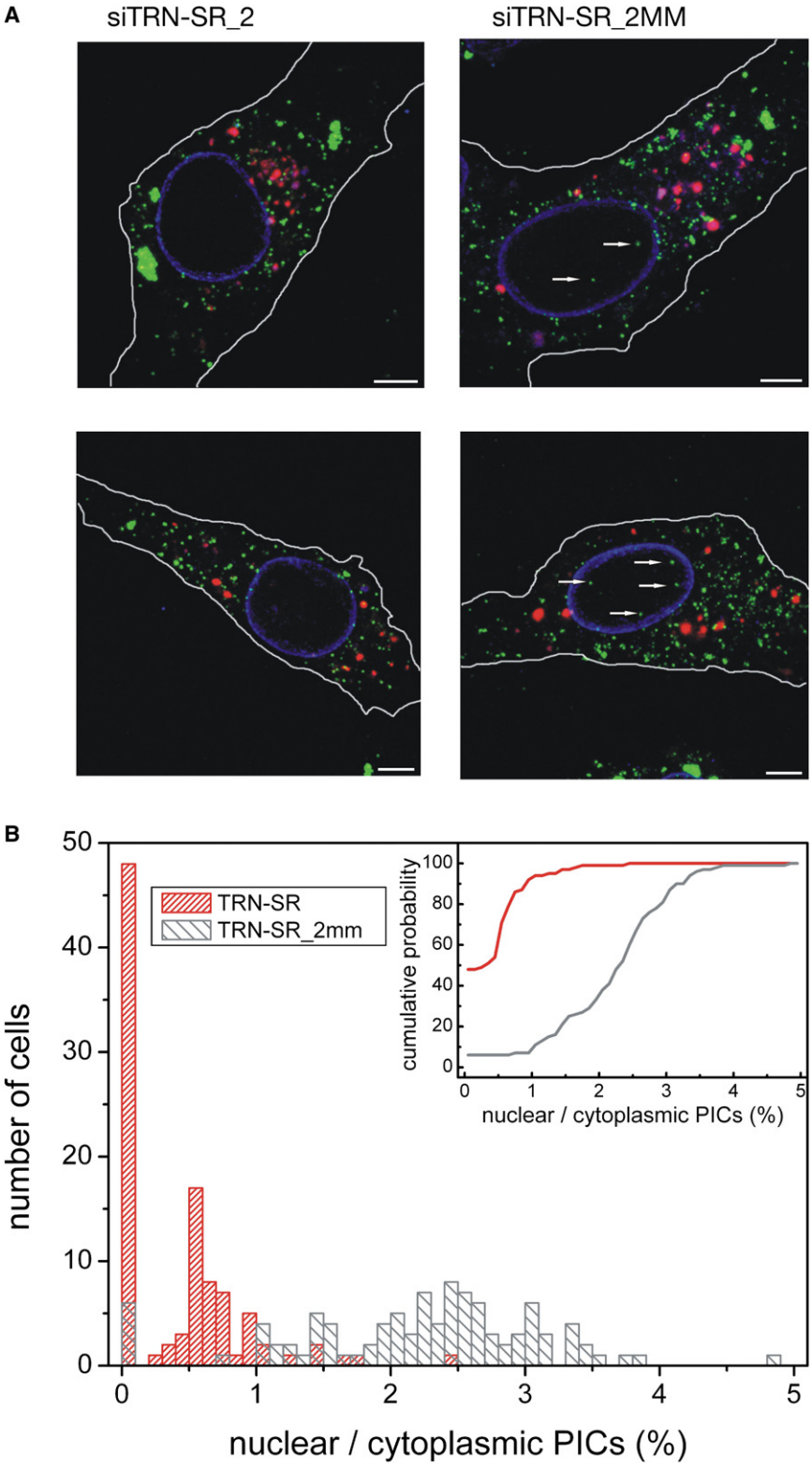

Figure 6. Nuclear Translocation of PICs in HeLaP4 TRN-SR2 Knockdown Cells

(A) Representative images of cells treated with Alexa-568 (red)-labeled siTRN-SR_2 (left) and siTRN-SR_2MM (right). Cells were infected with HIV-IN-eGFP (green) and immunostained with lamin A/C (blue). Six hours after infection, cells were fixed and analyzed by laser-scanning confocal microscopy. Images are derived from confocal Z stacks with the maximum projection of 3-μm-thick slices centered in the middle of the nucleus. Cell shapes are outlined in white. The white bars at the lower right corner of the images correspond to 1 μm. White arrows highlight the PICs observed in the nuclei.

(B) Percentage of PICs in the nucleus versus cytoplasmic PICs in cells treated with siTRN-SR_2 (red bars, n = 100) and siTRN-SR_2MM (gray bars, n = 100). Nearly half of the cells treated with siTRN-SR_2 did not contain any PICs in the nucleus, whereas in the mismatched control cells, on average 2.2% of the PICs had been imported into the nucleus. In the inset, the distribution of cumulative probabilities is plotted for TRN-SR2 siRNA-treated cells (red) and mismatched siRNA-treated cells (gray) ($p < 0.001$, Kolmogorov-Smirnov test).

integrase as the viral partner of interaction, nor could it validate TRN-SR2 as the nuclear-import factor of HIV, this independent identification lends further support to our study.

To productively infect host cells, HIV needs to perform two entry steps. First the virus has to attach to cellular membrane (co)receptors in order to enter the cytoplasm. After stripping the viral particle into the PIC, this multiprotein complex needs to dock onto nuclear-import receptors to cross the second physical barrier against infection: the nuclear membrane. Cell-fusion inhibitors like maraviroc and enfuvirtide have successfully entered the clinic (for a review of HIV drug development, see [44]). Because nuclear import is still poorly understood in comparison, it has remained a yet unexplored target in anti-HIV therapy. The strong replication defect provoked by silencing of TRN-SR2 provides a rationale for the identification of small-molecule protein-protein interaction inhibitors to obstruct loading of the PIC onto its nuclear-import factor. We believe that the interaction between IN and TRN-SR2 shows great promise as a novel target for future anti-HIV therapy.

Experimental Procedures

Recombinant-Protein Preparation

Recombinant proteins were expressed in *E. coli* BL21 (DE3). We kindly thank Dr. Woan-Yuh Tarn (Inst. of Biomedical Sciences, Taiwan) for the pGEX-TRN-SR2 expression plasmid. After transformation of the transportin expression plasmid, the bacteria were grown to an optical density of 0.4, and protein expression was induced by addition of a final concentration of 0.5 mM isopropyl-beta-D-thiogalactopyranoside. After incubation at 37°C for 4 hr, the bacteria were harvested and stored at −20°C until protein purification. For purification, the cells were resuspended in lysis buffer (PBS [pH 7.3], 1 mM DTT, 1 mM EDTA, 0.2 mM PMSF, 1 μl Benzonase/10 ml lysate). After complete lysis by ultrasonication, the supernatant was cleared by centrifugation and recombinant proteins bound to Glutathion Sepharose Resin (GE Healthcare, Belgium). After washing of the resin with 20 volumes of lysis buffer, the GST-tagged protein was eluted with 10 × 1 ml elution buffer, supplemented with 10 mM reduced glutathione (50 mM Tris/HCL,

pH 7.3, 500 mM NaCl). The fractions were analyzed by SDS-PAGE for protein content, pooled, and concentrated by dialysis (overnight, 4°C) against storage buffer (50 mM Tris/HCl, pH 7.3, 500 mM NaCl, 50 % [v/v] glycerol). Recombinant His_6-tagged HIV-1 integrase was purified as described previously [37]. Recombinant His_6-tagged MoMLV-integrase was purified as described previously [45].

Pull-down of TRN-SR2 from HeLaP4 Cells by Recombinant HIV-1 and MLV IN

Total HeLaP4 cell extracts were prepared as follows. Twenty Mio HeLaP4 cells were lysed in lysis buffer (20 mM Tris/HCl, pH 7.3, 400 mM NaCl, 0.2% [v/v] Triton X-100, 10% [w/v] sucrose, Complete Protease Inhibitor Cocktail [Roche, Germany]). The concentration of protein was determined by BCA Protein Determination Assay Kit (Perbio, USA). Two hundred micrograms of total protein was incubated with 3 or 6 μg of recombinant HIV-1 or MoMLV integrase on ice for 30 min in a final volume of 50 μl in order to allow interaction of the recombinant proteins with cellular interaction partners to occur. Subsequently, 50 μl of PD-buffer (50 mM Tris/HCl, pH 7.3, 200 mM NaCl, 0.05 % Nonidet P-40) was added prior to addition of 30 μl Ni-chelate-Sepharose (QIAGEN, Germany) equilibrated in PD buffer. Finally, 40 mM imidazole was added to prevent unspecific binding. Pull-down mixtures were incubated for 4 hr at 4°C before proteins bound to the Ni-chelate beads were collected by centrifugation (1000 RPM, 1 min, 4°C). The beads were washed twice with PD buffer supplemented with 80 mM imidazole. Then, proteins bound to the beads were eluted by addition of 4 μl imidazole (2 M) and SDS-page loading dye (160 mM Tris/HCl, pH 6.8, 2% [w/v] SDS, 200 mM DTT, 40 % glycerol, 0.1 % [w/v] bromphenol blue). Subsequently, the eluates were separated on a 12.5 % SDS-PAGE and proteins were visualized by western blotting.

Transient Knockdown of TRN-SR in Cell Culture

The day prior to transient transfection, 50,000 HeLaP4 cells were seeded per well in a 24-well plate for quantitative polymerase chain reaction (qPCR) analysis. For HIV-1 infection, 200,000 cells were seeded per well in a 6-well plate, and attachment to the plate was allowed overnight. For transient knockdown in macrophages, 200,000 macrophages in one well of a 24-well plate were used. Cells were transfected with 20 nM (HeLaP4) or 200 nM (macrophages) siRNA following the guidelines of the siFECTamin protocol (ICVEC, UK). The target sequences of the synthetic siRNA duplexes are shown in Figure 1. Synthetic siRNAs were designed as follows: siTRNSR_1, targeting nucleotides 352–371, 5′-GGAGCGCGCCUCUUUUUGGdTdT-3′ (QIAGEN, Netherlands); siTRNSR_2, targeting nucleotides 605–624, 5′-UCG GCGCACAGAAAUUAUAdTdT-3′ (QIAGEN, Netherlands); siTRNSR_2MM, targeting the same nucleotides, harboring four mutations (underlined), 5′-UCGGCGCAGUCUAAUUAUAdTdT-3′; siCD4, described previously, 5′-AAGATCAAGAGACTCCTCAGTdTdT-3′ [46]; and siGFP (siRNA libraries, Eurogentec, Belgium). For PIC nuclear-import assays, siRNA labeled with 3′ Alexa Fluor 546 were used. In parallel, a transfection without siRNA duplex was performed as a control and referred to as siMOCK-transfected.

HIV-1 Infection and Analysis of Transiently and Stably Transfected HeLaP4 and Macrophages

Three days after transient transfection, HeLaP4 cells were detached from the 6-well plates. Subsequently, 150,000 cells were brought into each well of a 24-well plate for 4 hr at 37°C. Of the stable cell lines, 150,000 cells were seeded into each well of a 24-well plate and incubated for 4 hr at 37°C. After attachment, cells were infected with HIV-1. Infections were performed with three amounts of virus as determined by p24 measurement (HIV-1 p24 ELISA kit, Perkin Elmer): 0.43×10^4, 1.1×10^4, and 2.2×10^4 pg p24 in a total volume of 250 μl. Each experiment was run in triplicate. After 3 hr of infection, the supernatant was removed and cells were washed three times with PBS prior to addition of 0.5 ml DMEM-complete. At 24 and 72 hr after infection of transient transfected cells, a single well was analyzed for β-galactosidase activity (chemiluminescent β-Gal Reporter Gene Assay, Roche Applied Science, Belgium). For the measurement of β-galactosidase activity, care was taken to measure reporter activity in adherent and detached cells. β-galactosidase activity was measured following the protocol supplied by the manufacturer. Chemiluminescence was measured with the Glomax (Promega, Belgium). The protein concentration of each sample was determined (BCA Protein Assay Kit, Perbio), and read-outs were normalized to 1 mg/ml total protein. At each time point analyzed, the persistence of knockdown was demonstrated by quantitative PCR or western-blot analysis. For the stable cell lines, aliquots of the supernatant were analyzed by p24 measurements 48 hr and 72 hr after infection (HIV-1 p24 ELISA kit, Perkin Elmer). Infections of macrophages were performed with 1.6×10^6 pg p24 in 500 μl RPMI-complete. After overnight infection, the medium was replenished. HIV-1 replication was monitored by p24 measurements 72, 144, and 192 hr after infection.

In Vivo PIC Nuclear-Import Assay

The HIV-IN-eGFP virus was obtained by transincorporation. In brief, 3×10^6 293T cells were transfected by calcium phosphate with 6 μg of pVpr-IN-EGFP (Vpr and IN cloned into the pEGFP-N1 vector, Clontech Laboratories), 6 μg of pD64E (pNL4-3 clone containing the IN D64E inactivating mutation obtained from the AIDS Reference and Reagent Program), and 1 μg of pVSV-G. Supernatants were collected after 48 hr, filtered through a 0.45 μm pore-size filter, and then concentrated by ultracentrifugation. Viral inocula equivalent to 3 μg of HIV-1-p24 were used to infect 40,000 cells. Two hours after infection, cells were incubated with trypsin (1 min) and fixed with 2% paraformaldehyde. Three-dimensional stacks of fixed cells were acquired with the TCS SL laser-scanning confocal microscope (Leica Microsystems) equipped with galvanometric stage and a 63 ×/1.4 NA HCX PL APO oil-immersion objective. Z step size was 0.3 μm. Ar and HeNe laser lines were used for EGFP (λ = 488 nm), Alexa-568 (λ = 514 nm), and Alexa-680 (λ = 633 nm) excitation. Fluorescence emission was collected in the ranges 495–511, 556–640, and 644–795 nm for EGFP, Alexa-568, and Alexa-680, respectively. For the two- and three-color analysis, a sequential image acquisition was used to reduce crosstalk between different signals below 5%. Multichannel images were contrast stretched (linearly) and assembled in ImageJ (NIH).

Supplemental Data

Supplemental Data include Supplemental Experimental Procedures and two figures and can be found with this article online at http://www.current-biology.com/cgi/content/full/18/16/1192/DC1/.

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